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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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SPIRAL BODIES IN BACTERIAL CULTURES

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During the winter of 1918–1919, in the course of some work with spore-bearing bacteria, spiral bodies resembling spirochetes were frequently found in cultures. When these were shown to Dr. Theobald Smith, he suggested that they were clusters of detached flagella such as had been seen by him at different times in cultures of anaerobes. Two interesting coincidences occurred at this time. The first was the receipt from an Institution of photographs of similar spiral bodies with the suggestion that they might be *Vibrio fetus* of infectious abortion in cattle, and the second was the publication of an investigation into spiral bodies in bacterial cultures by Koga and Otsubo (1919a). Since these authors have discussed the phenomenon as one hitherto undescribed and since their publication has more recently (1919b) appeared in Japan, it seems appropriate to call attention to earlier references and describe briefly the conditions under which these spiral bodies have now been found.

Loeffler (1889) first saw these spiral bodies when staining the flagella of the typhoid bacillus and the potato bacillus, but did not recognize their true nature until a year later (1890), when he found them in three different blood serum cultures of the bacillus of black leg. The latter were much larger than those seen in the typhoid cultures and he published, along with the description, a photograph of the preparation. At the same time he referred to a microphotograph, published by Fränkel and Pfeiffer, of the bacillus of malignant oedema in which spiral threads passed out from the organism just as he had found them in the typhoid bacillus. Three years later Sakharoff (1893) described and photo-

graphed spiral bodies found in cultures of an anaerobe, *Bacillus asiaticus*, isolated by him from stools of cholera patients and grown in gelatin stab cultures. He agreed with the hypothesis of Loeffler that these were made up of clusters of detached flagella, since they could not be gotten rid of in long series of transfers and varied considerably in length and thickness. Also they could not be evolution forms of the bacteria, as dead spirals were numerous in twenty-four hour cultures. In the same year Moore (1893) wrote,

> In the microscopical examination of well-executed preparations for exhibiting the flagella three conditions have been universally observed: (1) . . . . ; (2) there were a considerable number of detached or free flagella lying between the bacteria; and (3) the numbers of flagella on the different bacilli were not constant.

A more detailed account of these spiral bodies, also illustrated by photographs, was published by Novy (1894), when he described a new anaerobic bacillus of malignant oedema. He first found them unstained in smears stained with Gentian violet of the peritoneal fluid of guinea pigs and rabbits dead from inoculation, and then well stained in smears prepared after Loeffler's method. He found identical spirals in pure cultures of the organism and thought the nature of the media to be in some way connected with their formation. They occurred rarely in bouillon cultures. They were more numerous in gelatin cultures, most plentiful in agar cultures, and in the two last cases they were found in the condensation water. Loeffler's work, but not that of Sakharoff, was known to Novy and he confirmed the presence of these spirals in cultures of the bacillus of black leg and found them also in cultures of the bacillus of malignant oedema and of tetanus. He was not, however, like Loeffler convinced that they were clusters of flagella and he suggested the possibility of their being single deformed flagella, analogous to the involution forms found among bacteria, and named them "Riesengeisseln."

When studying the morphology of the tetanus bacillus Kanthack and Connell (1897) found two types of flagella which they named primary and secondary. Photographs of the latter show them

to be similar to the spiral bodies under discussion, but the authors found them always attached to the organisms.

Malvoz (1902) working with Wathelet found spiral bodies in a culture of *Bacterium coli* isolated from the stools of a typhoid patient. Preparations stained after Loeffler's method were shown by them to Nuel (1893) who considered them identical with spirals which he had found almost ten years earlier in cultures made from a bacterial disease of the cornea. Like Novy he regarded them as individual flagella calling them "cils géants." Malvoz, however, inclined to Loeffler's view and referred to Migula's description of their formation as the best. He called them "cils composées," following the terminology of Sakharoff in preference to that of Nuel.

That these spirals were known to bacteriologists at the end of the last century is proved not only by Flügge's (1896) brief reference to them and Migula's (1897) account of their formation, but by the remark of Zettnow (1899), "Geisselzöpfe habe ich in den jungen anaëroben Culturen nicht beobachtet." In the atlas accompanying the first edition of Kolle and Wassermann (1902) were published Zettnow's photographs of small tufts of flagella from a pure culture of an unknown bacterium and of a very large tuft of flagella from *Sarcina agilis*. More recent references may be found in the texts of von Hibler (1908), of Kolle and Wassermann (1912), and of Friedberger and Pfeiffer (1919).

Koga and Otsubo (1919) while attempting to get pure cultures of smegma spirochetes, found spirochete-like spiral bodies in cultures of saprophytic bacilli. The occurrence of such forms in bacterial cultures was evidently unknown to them and the result of their investigation led them to conclude that they were "nothing more than an unusual development of the flagella or parts of the bacterial bodies." They worked chiefly with *Bacillus subtilis* but gave a list of other organisms, in cultures of which spiral bodies were also found.

For a number of years it has been the custom in this laboratory to keep certain cultures in tubes closed with sealing wax. In order to find out the general effect on culture growth of such a method a series of experiments with a number of spore-bearers was begun

during the winter of 1918–1919. In the cultures of motile forms, viz., *Bacillus cereus*, *Bacillus mesentericus-fuscus*, *Bacillus mesentericus-vulgatus* (2 strains), and a bacillus isolated from the lung of a calf and designated "Organism A," non-motile spiral bodies resembling spirochetes were frequently seen. None such were found in the cultures of non-motile forms being studied. All the cultures were grown on plain agar slants and the tubes were closed immediately after inoculation with paraffin-dipped cotton stoppers cut off level with the top of the tube and then pushed down approximately $\frac{1}{16}$ inch below the top. The mouth of the tube was flamed until thoroughly hot, when a small amount of sealing wax was placed over the stopper. This was absorbed by stopper and, when the tube had cooled, the space above the stopper was filled with sealing wax care being taken to leave no air bubbles. Several series of cultures were also grown in bouillon sealed in the same way as the agar.

Spiral bodies were found in cultures of all five organisms, but they appeared with greatest regularity in the two strains of *Bacillus mesentericus-vulgatus*, in cultures of which Loeffler (1889) had also found them. At first it was thought that they were to be found only in sealed tubes, but later it was discovered that they were always present in the condensation water of twenty to twenty-four hour unsealed cultures of *Bacillus mesentericus-vulgatus*. They were also found in unsealed cultures of *Bacillus mesentericus-fuscus* and *Bacillus cereus* after the third day. They were seen first in hanging drops made from the condensation water, but were not found in smears made from the same and stained with methylene blue or carbol fuchsin. However, in smears stained after Loeffler's method they were always found well stained; but, if Johnston and Mack's modified method was followed they were not found, doubtless because they had disintegrated during the prolonged incubation in sterile water. Even in young cultures spirals of different sizes were seen, but very large ones, similar to those of the bacillus of black leg photographed by Loeffler, were found only in sealed cultures after an incubation period of fourteen to twenty days. In these large spirals striations parallel to their longitudinal axis were frequently seen. Their

non-motility and reaction towards stains differentiated them definitely from true spirochetes and their presence, in cultures of motile organisms only, suggested a relationship with the flagella. Further, their regular absence from preparations stained after Johnston and Mack would seem to prove that they were lifeless.

DISCUSSION

Since this phenomenon has been most frequently seen during the investigation of anaerobes, it has been thought that anaerobiosis and the formation of spiral bodies were in some way connected. It is now evident, however, that they are formed in aerobic cultures. We have found them exclusively in the condensation water of such cultures and in its rapid drying out may rest the explanation of their having been so frequently missed. Sakharoff (1893) when studying an aerobe, found them in hanging drops of the liquefied gelatin and there as in *Bacillus mesentericus-vulgatus* a stout pellicle had grown over the surface of the liquid. It may be said that under such a pellicle anaerobic conditions exist, but spiral bodies were found in the condensation water of *Bacillus cereus* and *Bacillus mesentericus-fuscus* after the third day, when no pellicles had formed. Also, both Loeffler (1890) and Moore (1893) found them in stained preparations of the typhoid bacillus made from cultures which they do not say were grown anaerobically. Koga and Otsubo (1919) found them in cultures of a number of bacilli but all were cultivated anaerobically. They further claim to have found flagella on *Pfeifferella mallei* and spiral bodies in their anaerobic cultures of this form.

In the earliest references no emphasis was laid on the nature of the media on which the organisms were cultivated. Novy (1894) was the first to suggest a relationship between the media and the formation of spiral bodies. Our findings agree with his in that spiral bodies were most abundant in the condensation water of cultures grown on agar slants and were very rare or entirely absent in bouillon cultures. We have not used gelatin. Koga and Otsubo (1919) state that spiral bodies did not develop at all in media lacking fresh protein and were never found, when

the organisms were cultivated on agar. In explanation of these opposite findings it may be suggested that the Japanese workers studied only the colonies on the agar slants, in which we have never found spiral bodies, and did not examine the contents of the condensation water, where we have repeatedly found them. These authors did not specify in what part of the cultures grown on media containing fresh protein the spiral bodies were found, but Loeffler (1890) described those found in the cultures of the black leg bacillus as lying on the surface layer of the blood serum.

To Migula's (1897) description of their formation as a mechanical process resulting from the collision of motile bacteria and the intertwining of their flagella in a circumscribed space nothing definite can be added. It is possible, however, that the nature of the media on which an organism is grown may exert a secondary influence on their formation and may explain the variation in their time of appearance in cultures of different organisms. It was thought that the viscosity of the condensation water might influence the formation of spirals, but this proved not to be the case.

SUMMARY

Spiral bodies resembling spirochetes were found in cultures of bacterial organisms grown aerobically, as well as in those grown in a limited amount of oxygen. They were regularly present in the condensation of water of such cultures.

As has been pointed out by other workers, such spiral bodies are to be distinguished from spirochetes (1) by their lack of motility,(2) by their reaction towards stains, and (3) by the impossibility of obtaining them in pure culture.

Their relationship with flagella is further proved by their presence in cultures of motile organisms only.

Their disintegration when incubated for two to three days in sterile water is evidence of their lifelessness.

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THE CAUSE OF EYES AND CHARACTERISTIC FLAVOR IN EMMENTAL OR SWISS CHEESE[1]

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INTRODUCTION

Due to a lack of the proper natural inoculation in the milk, the Swiss or Emmental cheese industry in the United States has had only a limited success. The peculiar sweetish flavor which is characteristic of the best cheese of this type is very commonly lacking in our American-made cheese. It is also frequently deficient in eye development, and in fact in some cases the cheeses are entirely "blind." Swiss cheese is made only during certain seasons in America, because of the uncertainty of obtaining the proper development of eyes and flavor. It would seem that this industry could be put on a sounder as well as a more scientific basis by the use of cultures which would cause proper ripening in the cheese. With such cultures at hand it should be possible to make Swiss cheese of a uniform and high-grade quality throughout the year; such practice should result in raising very materially the quality, as well as the quantity, of our American-made Swiss cheese.

Von Freudenrich and Orla-Jensen (1906) in their work in Switzerland have isolated propionic acid-producing bacteria which they consider the cause of eyes in Emmental cheese. The essential organism, called by them *Bact. acidi-propionici* (*a*), was found to ferment lactates with the production of propionic acid, acetic acid, and carbon dioxide. Other varieties of propionic

[1] Published with the permission of the Secretary of Agriculture.

bacteria were found but they did not appear to have much influence on the ripening of cheese.

In the early experiments conducted by the Department of Agriculture on Swiss cheese, some cultures of propionic acid bacteria were obtained from Professor Burri of Berne in the hope that these could be introduced and used in the manufacture of Swiss cheese in this country. The experiments conducted with these cultures, however, were not encouraging; in fact it was not established experimentally that they were able to cause the development of eyes when used for starters in the manufacture of Swiss cheese. Following the methods of Von Freudenrich and Orla-Jensen, cultures were isolated which corresponded to their published descriptions of the propionic-acid bacteria. These were also used in the manufacture of experimental Swiss cheese with negative results. These findings do not discredit the work of Von Freudenrich and Orla-Jensen, since it is entirely possible that the cultures used belonged to varieties which do not play important rôles in the ripening of Emmental cheese. The experiments referred to were carried on a few years after the death of Professor von Freudenrich; Professor Orla-Jensen at that time was not able to furnish cultures of these organisms.

I. CONCERNING THE OCCURRENCE AND NUMBERS OF LACTATE-FERMENTING BACTERIA IN EMMENTAL CHEESE

That there exist in Emmental or Swiss cheese bacteria which ferment lactates with the production of volatile acids has been shown by Von Freudenrich and Orla-Jensen (1906), who succeeded in isolating such organisms in pure culture; and the theory was advanced that the production of eyes is due to the carbon dioxide liberated by these bacteria in the transformation of lactic acid to propionic and acetic acids, according to the formula:

$$3\ C_3H_6O_3 = 2C_3H_6O_2 + C_2H_4O_2 + CO_2 + H_2O.$$

They also determined the approximate number of lactate-fermenting organisms in Emmental cheese by means of dilution cultures in a calcium lactate broth. By such methods they were

able to demonstrate that these bacteria occur in numbers from 10,000 to 200,000 per gram of cheese.

Troili-Petersson (1909) using the same methods found approximately the same numbers of lactate-fermenting bacteria as did Von Freudenrich and Orla-Jensen. In a previous report from these laboratories, Eldredge and Rogers (1914), who worked with American cheese of the Emmental type, found this type of organism present in somewhat smaller numbers than was reported by the European workers, and in fact apparently entirely lacking in some cheese.

Modification of the lactate broth of Von Freudenrich and Orla-Jensen

For the growth and isolation of lactate-fermenting bacteria from Emmental cheese, Von Freudenrich and Orla-Jensen (1906) used a calcium lactate broth of the following composition:

	per cent
Pepton (Witte)	2.0
Sodium chloride	0.5
Dipotassium phosphate	0.2
Calcium lactate	2.0

Although such a mixture is obviously faulty, due to the incompatibility of the calcium and phosphate ingredients, resulting in a heavy precipitate of an insoluble calcium phosphate upon sterilization, the broth as used by Von Freudenrich and Orla-Jensen, so far as we are aware, has not been modified by subsequent workers who have used it extensively for studies of the propionic and butyric acid-forming groups of bacteria. Only recently Boekhout and De Vries (1917) have employed it in an extensive study of the bacteria responsible for gas formation in cheese.

It need hardly be mentioned, assuming that the several components of the broth are in fact of value, that the ingredients added should not be rendered inert by precipitation. This may be obviated by the use of another salt of lactic acid, such as sodium lactate, in place of the calcium. The commercial sodium lactate syrup may be used if desired, but we have found it convenient to prepare the sodium lactate just before use by neutralizing the desired amount of lactic acid with sodium hydroxide. The sodium lactate broth has been found to be in all respects as good

as that made with the calcium salt, and in one very important respect to be superior.

In the preparation of the calcium lactate broth no attention, so far as the published papers indicate, has been paid to its reaction; and considering the reactions of the individual ingredients employed there would seem offhand to be little need for concern about this point. It was noted, however, when broth was tested for its hydrogen-ion concentration, by means of the colorimetric method of Clark and Lubs, (1917) that a value of about pH = 5.2 was always obtained. This result (from pH = 5.1 to pH = 5.3) was found with Witte pepton as well as with a variety of American brands.

An inquiry was therefore made into the reactions of the individual components and of combinations of the several components. Calcium lactate broth was made and at the same time solutions of the various ingredients were prepared separately in the same concentrations as they occur in the broth. These were all sterilized in the autoclave for twenty minutes at 15 pounds pressure. After cooling, the following results were obtained:

	pH
2.0 per cent pepton	6.8
0.5 per cent sodium chloride	7.3
0.2 per cent dipotassium phosphate	8.2
2.0 per cent calcium lactate	7.3
Von Freudenrich and Orla-Jensen broth	5.2

As is shown by these data, the reaction of the finished broth is much more acid than is any one of its several components. Another lot of broth made with the same ingredients, with the exception that sodium lactate was substituted for the calcium salt, gave a reaction of pH = 7.2.

It would appear then that the explanation is to be found in the reaction between the phosphate and the calcium lactate; and such it seems is the case, as is indicated by the result given below. These solutions were sterilized as were those reported above.

	pH
0.2 per cent dipotassium phosphate	8.2
2.0 per cent calcium lactate	7.3
0.2 per cent dipotassium phosphate + 2 per cent calcium lactate	4.8

The marked acidity of the lactate-phosphate mixture is probably explained by the formation of acid phosphates and lactic acid along with the insoluble calcium phosphate.

Aside from the case of this particular broth, the principle here illustrated should be given more general consideration in the formulation of culture media. It would seem, *à priori*, that there is danger of such a shift in the hydrogen-ion concentration upon sterilization of any medium which contains calcium or magnesium and a phosphate, if the calcium-magnesium portion is in excess of the phosphate. This principle is violated in many of the synthetic media which are recorded in bacteriological literature. It is obvious also that the buffering effect of the phosphate is lost in such a combination.

In the recommendation that sodium lactate be used in the place of the calcium salt, it has been assumed that the dibasic phosphate employed in this medium serves some useful purpose. As a matter of fact, in pure culture, the lactate-fermenting bacteria of Swiss cheese grow very well in broth containing only pepton and either calcium or sodium lactate. This does not prove that the simplified medium would be just as good for quantitative estimations in which the seedings are very light.

In the work here reported quantitative determinations were made in a broth containing 1 per cent pepton, 1 per cent dried yeast and 1 per cent lactic acid (as sodium lactate). This broth supports a very active growth of the lactate-fermenters and is an excellent one for quantitative purposes.

Approximate numbers found

Quantitative dilutions of cheese were planted in broth composed of 1 per cent each of pepton, dried yeast, and lactic acid (in the form of sodium lactate). Dilutions of from 0.01 to 0.000,001 gram of cheese were tested. After incubation for four weeks at 30°C. the cultures were acidulated and subjected to steam distillation to determine the formation of volatile acids. Control flasks containing pepton-yeast broth without the lactate, inoculated with the same dilutions, were run in order to avoid any possible error through the measurement of the relatively small amounts of vola-

tile acids derived from the nitrogenous constituents of the medium.

Without going into details, it may be stated that of 16 samples of American-made Swiss cheese purchased on the open market all contained lactate-fermenting organisms in sufficient numbers to be revealed in 0.000,001 gram, the highest dilution used. These samples were representative of about the average run of domestic Swiss cheese; only samples which had sufficient eye development were taken, but the flavor varied from excellent to very poor.

Thus it will be seen that we have succeeded in demonstrating the presence of lactate-fermenting organisms in numbers considerably greater than has been reported by other investigators. Also, as will be shown later on, these bacteria have been isolated directly from cheese without previous enrichment in some selective broth.

Relation to previous work

Concerning the discrepancies between the results of various workers on this subject, we feel that these inconsistencies may well be reconciled through the observations made in connection with the work here reported. As has been noted, the reaction of the lactate broth, as employed by Von Freudenrich and Orla-Jensen and subsequent workers, is too acid for the best results. The error which may be introduced by this factor is well illustrated by the following test made on a pure culture of a lactate-fermenting organism from Swiss cheese: A broth culture one week old as tested by the dilution method, using the regular Von Freudenrich and Orla-Jensen broth (pH = 5.2), and another broth of the same composition except that sodium lactate was substituted for the calcium salt. This broth had a reaction of pH = 6.8. The result of this test showed that, whereas the sodium lactate broth gave a count of over 100,000,000 organisms per cubic centimeter the number as indicated by the calcium lactate broth was less than 1,000,000.

Aside from the error introduced through the unfavorable reaction of the calcium lactate broth, as it has been previously used, there are apparently other factors which make the dilution method

a rather uncertain one for the quantitative estimation of the lactate-fermenting bacteria of cheese. It has been noted on several occasions that the distillation for volatile acids gave negative results whereas further propagations from the culture used showed that lactate-fermenting bacteria were present. This phenomenon is probably to be explained by the presence in the culture of other organisms which consume the volatile acids. That this may sometimes be the case was indicated by the results obtained on certain samples of cheese in which volatile acids were produced from the high dilutions of cheese in lactate broth whereas the low dilutions, which contained a much heavier inoculation and a greater variety of organisms, gave negative results. In this work we have checked ourselves quite thoroughly against such errors by running all of our dilution cultures in triplicate, and also by making further examinations and propagations from dilution cultures which gave negative results. Thus we have on several occasions demonstrated the presence of the lactate-fermenting bacteria from cultures which gave negative results on the original test.

II. THE CAUSE OF EYES AND FLAVOR

In our work on Swiss cheese during the past few years the identity of the organism responsible for the development of the characteristic flavor, as well as the eyes, of Emmental cheese has been quite clearly established. The ability of this organism to play these rôles in the ripening of cheese has not only been established by carefully controlled laboratory experiments, but also under practical commercial conditions in factories located in widely separated areas of the country.

GENERAL CHARACTERISTICS

Morphologically the organism is a minute rod about twice as long as it is broad. It makes little or no growth on agar slope cultures; in agar stabs growth takes place throughout the length of the puncture but not on the surface. In agar shake cultures there is likewise no growth on the surface whereas good growth takes place throughout the medium; as incubation continues over

an extended period the growth is seen to become very heavy, barely below the surface of the agar. In a suitable nutrient broth a heavy slimy growth occurs at the bottom and the whole broth becomes turbid, with the usual exception of a narrow clear zone at the surface. Milk is rendered slowly acid and is usually curdled in from one to two weeks at 30°C. Growth in pepton milk is much better, curdling taking place in from four days to one week at 30°C. Small bubbles of gas may sometimes be seen in the curd. Gelatin is not liquefied. Glucose, lactose, maltose, sucrose, glycerol and salicin are fermented; raffinose, inulin, and mannitol are not.

One of the outstanding characteristics of this organism is the production of a large amount of catalase. Attention has previously been called to the relatively large amount of catalase which is found in Swiss cheese (Sherman 1919). The group of organisms herein described is the one which was shown to give this charteristic to cheese of the Emmental type.

Reference to the products produced by this bacterium indicates that it belongs to the group of proprionic acid bacteria which was described by Von Freudenrich and Orla-Jensen. Lactates are fermented with the production of volatile acids, including propionic and acetic, and carbon dioxide. Also in the fermentation of lactose, volatile acids and carbon dioxide are produced.

Relation to previously described types

Whether this organism is identical with any of the types isolated by Von Freudenrich and Orla-Jensen cannot be definitely stated at this time. In general it appears to agree quite closely with their description of *Bact. acidi-propionici* (*a*) which they considered to be the true cause of the development of eyes in cheese. A few points, however, in their description do not agree with the characteristics which we have observed in our organism; they state that it causes no visible change in milk, whereas our organism in litmus milk develops an acid reaction after several days and causes coagulation on longer incubation. Further, from their results on the production of volatile acids it was noted that only a small amount of these substances was produced from glycerol,

while our organism causes an active fermentation of glycerol with the production of a considerable quantity of volatile acids. Finally, it may be stated that the organism with which we have been working causes the typical sweetish flavor in Emmental cheese, whereas the experiments of Von Freudenrich and Orla-Jensen did not give definite results on this point. Orla-Jensen (1912) has since stated more conclusively that the sweetish flavor is due to a factor other than the propionic acid bacteria.

It is of course recognized that an accurate comparison can not be made from published descriptions. It is hoped, therefore, that we may obtain from European workers cultures of the various types of propionic acid-forming bacteria so as to determine more definitely whether this organism is identical with any of the previously described types or whether it is a new variety. In this connection it may be noted that we have also isolated a variety of these lactate-fermenting bacteria, among which have been found quite distinct types. Although the characteristics of all of these varieties have not been studied in detail, they appear to agree in a general way with the types which have been isolated by European workers. It is hoped that further studies on these organisms, in comparison with types obtained from Europe, may be made in the future.

In keeping with the nomenclature used by the European workers for the group of propionic acid-producing bacteria, this organism will be tentatively designated as *Bact. acidi-propionici* (*d*).

The isolation of cultures

The direct isolation of this organism from cheese is difficult for various reasons, particularly because of its slow growth and its oxygen requirements. Though not a strict anaerobe it requires a considerably reduced oxygen tension. Although in pure culture this organism grows in all ordinary culture media, including even 1 per cent pepton solution, it is apparently not so easy to obtain growths from it when taken directly from cheese. On a few occasions colonies have been isolated

from agar plates made directly from cheese, but success by this method is rare.

On a number of occasions this organism has been isolated, directly from the cheese, by sealing agar dilutions in glass tubing of about 0.5 cm. diameter. With this method it is very easy to isolate the individual colonies by cutting the tube at the desired points. By sterilization of the outsides of these tubes by immersion in a strong disinfectant solution, and then rinsing with sterile water, there has been no difficulty in making isolations by this method without contamination. The medium which we have found very satisfactory for this purpose is one consisting of 2 per cent pepton, 1 per cent yeast, 1 per cent lactic acid (as sodium lactate) and 1.5 per cent agar. Although we have had fairly good success in making isolations by this method, it has by no means always proven successful.

By making enrichment cultures of the cheese in lactate pepton broth, as was done by Von Freudenrich and Orla-Jensen, the isolation of lactate-fermenting bacteria is much easier. We have isolated a variety of organisms belonging to this group from such enrichment cultures.

Our interest thus far has been more in the practical application of these bacteria in the cheese industry than in making a thorough study of their physiological characteristics. There is little doubt however, that by taking advantage of their known properties, a simple differential method could be developed which would be satisfactory for the direct isolation of this group of organisms from cheese.

Rôle in cheese

For studying the effect of this organism in cheese we have had at our disposal a supply of milk, obtained from the experimental herd of the Dairy Division, which was entirely lacking in the bacteria necessary for the development of the desired characteristics of Emmental or Swiss cheese. Cheese made from this milk by the Swiss method is always entirely lacking in the characteristic sweetish flavor, and is also frequently "blind." When the natural inoculation in this milk is such as

to cause a development of eyes in the cheese the resulting flavor is in no way similar to that characteristic of the typical Swiss cheese. This fact is important, since it shows that the formation of eyes may be due to bacteria other than the one herein described; it probably explains also the fact that American Swiss cheese is so frequently deficient in flavor even when abundant eye formation takes place.

In our laboratory work small cheeses of the Emmental type are made from about 200 pounds of milk. These cheeses are then handled in exactly the same way as are the large Swiss cheeses made under factory conditions, and they ripen in an entirely normal manner. From such experiments it has been demonstrated time and again that the organism described in this paper is responsible for the characteristic sweetish flavor of Swiss cheese and that it also causes the development of eyes. Its relation to the eye formation is shown in the photograph reproduced at the end of this paper; its relation to flavor production has been demonstrated in over 100 laboratory experiments in which one cheese in each experiment was inoculated while another cheese made from the same milk was left uninoculated.

That the use of this bacterium as a "starter" is practicable under commercial conditions has been demonstrated in a number of different factories. In all cases these factory experiments have shown a marked influence on the ripening of the cheese with respect to both eyes and flavor. The application of these results in cheese-factory practice will be treated more in detail in a future publication.

ACKNOWLEDGMENT

Should the work herein reported prove of value to the cheese industry, major credit therefor is due Mr. L. A. Rogers, in charge of the Research Laboratories of the Dairy Division, who recognized the possibilities of pure cultures in the manufacture of Swiss cheese and initiated work toward that end over ten years ago, and who has fostered and directed the work through

a period of many discouragements due to lack of facilities and frequent changes in the experimenting staff.

Acknowledgment is also due to Mr. K. J. Matheson, and his several collaborators, whose cordial coöperation in conducting the cheese-manufacturing tests has made this work possible.

SUMMARY

1. Bacteria capable of fermenting lactates with the production of volatile acids have been found to be constantly present in normal cheese of the Swiss or Emmental type in numbers exceeding 1,000,000 per gram.

The discrepancies in the results of previous workers on this subject are probably explained by a faulty combination of salts contained in the medium used, resulting in the production of a reaction too acid for the optimum development of the organisms concerned.

2. The essential organism for the production of eyes and the characteristic sweetish flavor of Swiss cheese has been isolated and studied.

The organism concerned belongs to the group of propionic acid-producing bacteria, but appears to differ slightly in some of its characters from the several varieties of propionic bacteria which have been described in the literature.

Factory experiments have shown that pure cultures of the organism may be used successfully in practice to insure the proper ripening of Emmental cheese.

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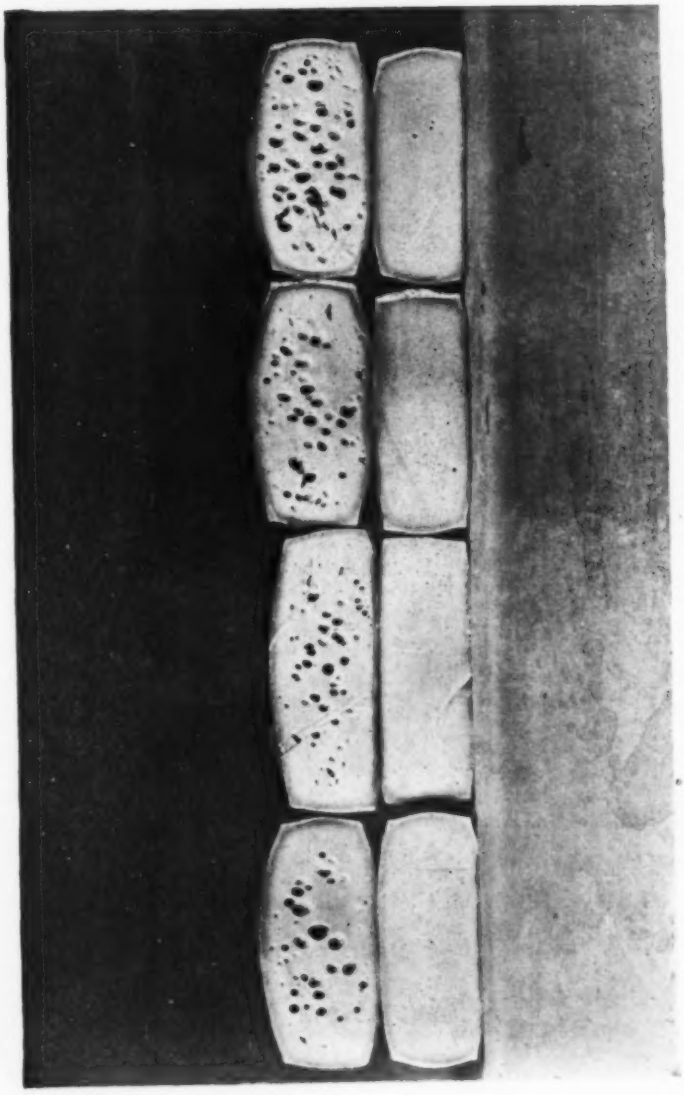
PLATE 1

The lower row of cheeses were made from milk lacking in the bacteria essential for the proper ripening of Swiss cheese.

The cheeses in the upper row were made from the same milk as their respective "blind" controls, with the addition of a pure culture of the eye and flavor producing organism.



A NEW MODIFICATION AND APPLICATION OF THE GRAM STAIN

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In making microscopical examinations of the quality of milk received at New York state cheese factories a need arose for a stain which would have a greater differential value than methylene blue, and which would be applicable for quantitative as well as qualitative results. The thought of the Gram stain at once suggested itself. While organisms can be classified only into general groups in methylene blue preparations, and no differentiation can be made between desirable and undesirable types for cheese making, the gas forming groups can be readily distinguished from the desirable lactic acid organisms in slides stained by the Gram method.

In developing a modification of the Gram stain which could be used in staining milk smears, the difficulty has been to secure a decolorizing solution which would allow the Gram positive organisms to retain the stain and still remove the color from the milk and the Gram negative types. The following method has proved satisfactory in our work, and is presented with the hope that it will help solve similar difficulties for other investigators.

The stain is as follows:

Gentian violet solution

Anilin oil	3.0 cc.
Alcohol (absolute)	7.0 cc.
Water	90.0 cc.
Shake; filter	
Gentian violet (Grübler)	2.0 grams

Iodine solution

Iodine	1.0 gram
Potassium iodide	2.0 grams
Water	300.0 cc.

Decolorizing solution

Anilin oil (2 parts) Xylol (1 part)	mixture	5 parts
Alcohol (95 per cent)		95 parts

Counter stain

Bismarck brown	4.5 grams
Water (boiling)	50.0 cc.
Filter	
Alcohol (95 per cent)	30.0 cc.

The milk smears were prepared by the usual Breed method (Breed and Brew, 1916); i.e., depositing 0.01 cc. of milk on a clean glass slide and spreading with a needle over an area of 1 sq. cm. The smears were dried and placed in xylol until the fat was dissolved, removed, drained, and immersed in 95 per cent alcohol for two minutes for fixing. The slides before being allowed to dry were placed in the gentian violet for forty-five seconds, blotted or allowed to drain after removing from the stain, and immersed in Gram's iodine solution for one minute, destained in the anilin-xylol-alcohol solution until no more stain could be removed; and then counterstained in Bismarck brown for forty-five seconds.

Several formulas of gentian violet solution were used but the particular concentration given has yielded the most consistent and satisfactory results. Satisfactory preparations could not be obtained with "Method 1" (commonly known as Stirling modification) of the Report of the Committee on the Descriptive Chart of the Society of American Bacteriologists (Conn et al., 1919) as light blue and green areas were deposited on the slide when such concentrated gentian violet was used. This was especially true of smears prepared from milk which had developed any degree of acidity. This reaction was probably due to the conversion of the gentian violet into closely related dyes in the presence of the acid and the alcohol of the destaining solution. No definite data are available at present on this point. The stains used in all cases were Grübler's.

The addition of the anilin oil and xylol to the destaining alcohol resulted in retarding the action of the solution sufficiently

to allow the Gram positive organisms to retain the stain while the color was removed from the Gram negative bacteria and the background of milk. Hastings, Evans and Hart (1912), in their cheese work used a decolorizing solution of anilin oil one part and xylol two parts. This solution although removing the stain from the Gram negative organisms and the milk, was slow in action and caused the organisms to appear distended and less brilliant in the final preparation. Consistent results could not be obtained using alcohol as a decolorizer as the stain was removed from the Gram positive bacteria before the milk was sufficiently destained. The results obtained with acetone, as a decolorizer, were similar to those where alcohol was used.

With exception of Bismarck brown,[1] no counter stain exhibited sufficient range of affinity between the nucleo-proteins of the cells and the casein of the milk to allow for different intensities of color even if destained. A few successful smears were made where an aqueous solution of safranin was used as a counter-stain, provided the slides were well washed before the application of the safranin. A heavy precipitate will be deposited on the smear if the Bismarck brown is not frequently dissolved and filtered.

The above method has been used for the routine examination of milk samples for an entire season at a cheese factory where all grades of milk were being received, and it proved helpful in eliminating milk which would develop gassy curds. The smears were checked with duplicate samples stained with methylene blue and no appreciable difference in the count could be observed.

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[1] If Bismarck brown stains the background of milk too deeply, slide may be immersed for a few seconds in a weak aqueous solution of acid fuchsin, after counter staining.

COLOR STANDARDS FOR THE COLORIMETRIC MEASUREMENT OF H-ION CONCENTRATION

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Contribution from the Research Laboratory of Physical Chemistry of the Massachusetts Institute of Technology, No. 135

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In an article of the above title recently published in this journal, Medalia (1920) presents a system of color standards somewhat similar to one published by me a little before (Gillespie, 1920). The work is evidently independent of mine, but the proposed tables are in serious disagreement with the results of my work.

The cause of the disagreement apparently does not lie in a conflict of observations, but in the plan followed by Medalia in preparing the tables.

It is stated that a test of this plan with the indicator, bromthymol blue, "succeeded perfectly, i.e., the green color was found at (pair no. 4) pH 7; or slightly yellowish green at (pair no. 3) pH 6.8 according to this range. (The change of color of this indicator was found by the writer to start with pH 6.2 instead of pH 6 as given by Clark and Lubs.)"

Unfortunately, this test is not sufficient to afford evidence in favor of the plan as against the method used by me to "smooth out" experimental errors for the preparation of tables, because the mass action equation used for this smoothing requires that such a limited test of the plan shall succeed perfectly, the error involved being only 0.02 pH, well within the experimental error.

In fact, the mass action equation requires that, if one is able to determine both limits[1] equally distant from the half-transfor-

[1] Although there are practical limits to the useful range, there is of course no real point of pH where the indicator "starts in" to change color, but only a subjective point "over the threshold" where it may appear to do so.

mation point (at pair 4), then pairs 3, 4, and 5 will be substantially correct as calculated by the plan in question, but pairs 2 and 6 will be in error by nearly 0.10 pH, and pairs 1 and 7 by about 0.25. The mass action equation is, however, in accord over the useful interval of pH with the measurements of Tizard (1910) for methyl red, of Barnett and Chapman (1918) for phenol red,[2] and of the present writer for all indicators studied by Medalia, except the acid range of thymol blue, which was not studied.

We do not need to assume the applicability of the mass action equation in order to show that the proposed tables are in disagreement with these measurements. It is only necessary to plot the results to be compared on one diagram in any uniform manner,[3] and the discordance will be apparent. The proposed tables must therefore be considered incorrect, since the plan on which they are mainly based lacks a solid foundation, and is not supported by enough data to put into question the conflicting measurements.

In the article, mention is made of measurement of acid production of bacteria by means of pH determinations. A word of warning seems justified by the fact that the idea is apparent in the writings of others. The definition of acid production in terms of a difference between initial and final pH values is decidedly not superior to definition in terms of titration, but rather false, or at least of slender and involved significance. To measure *how much* acid is produced we must *titrate*.[4] If the composition of the culture medium makes impossible a true titration on the direct culture, then we may distil the volatile acids and titrate

[2] This has been shown by me (Gillespie, 1920).

[3] For instance, the percentage of indicator placed in the alkaline solution may be plotted against the pH pertaining to it, or better, the logarithm of the ratio between the quantities of indicator as distributed between the alkaline and the acid tubes of the color standards may be plotted against pH. By the second procedure a straight line is required by the mass action equation. Mathematically, the plan of Medalia consists of a pure guess as to the form of the curve obtained by such plotting of the data.

[4] Measurement of change of pH may, in some cases, give us the acid production, if we have already incorporated the results of titration in a titration curve.

them, or possibly change the composition of the culture medium ("standard methods" notwithstanding), or resort to even graver expedients, but the last expedient indeed should be the measurement of pH for the given purpose. Measurement of pH and titration furnish two distinct methods of attack, each with its own object and interpretation. The principles involved have been carefully discussed by Clark and Lubs (1917).

As to a statement to the effect that the electrometric method is more accurate than the colorimetric, but that the apparatus which it requires is beyond the possibilities of the average bacteriological laboratory; the writer can subscribe to neither part in the unqualified form, but would refer again to the article of Clark and Lubs (1917) for a discussion of the first part, and to the recent book of Clark (1920) for the second. The writings of Clark and Lubs also contain full discussions of other principal topics, such as titration of culture media, effect of bacterial growth and of sterilization upon the indicators, etc.

It is pleasing to note that Medalia was able to preserve his color standards. The standards prepared by me were not permanent, and the main difference seems to be in the means taken by Medalia to avoid microbial decomposition, this point having been neglected by me.

It seems well to describe in this article, otherwise not very constructive, an instrument for further study of the indicator constants and behavior, which was devised too late to be of service in the work published (Gillespie, 1920). The necessary improvements in method, for work substantially better than that already published, must include temperature control of the buffer solutions in which the indicator is placed, and more precise measurements of the percentage transformation. The apparatus shown schematically in figure 1 can easily be made to satisfy both requirements. The writer has not seen it described. It is a colorimeter for two-colored indicators, and by an obvious modification it can be used to determine, if desirable, both the percentages of the two colors present and the total concentration. A simple apparatus was improvised[5] with which the percentages

[5] In the Laboratories of Soil Fertility, Bureau of Plant Industry, Washington, D. C.

could be determined with far greater ease and precision than is possible with a one-colored indicator in the usual colorimeter, since the quality changes very rapidly with the adjustment. Plane polished surfaces are desirable in the optical system, but were not used.

The glass vessels *A* and *C* are fixed in position, and *B* can be moved up or down, the motion being measured by a pointer (not shown) fixed to *B* and moving upon a scale divided into 100 parts. The instrument is so made that the pointer moves from 0 to 100 when *B* moves from contact with *C* to contact with *A*. The acidified indicator solution of suitable strength may be placed in *B* and an alkaline indicator solution of the same strength

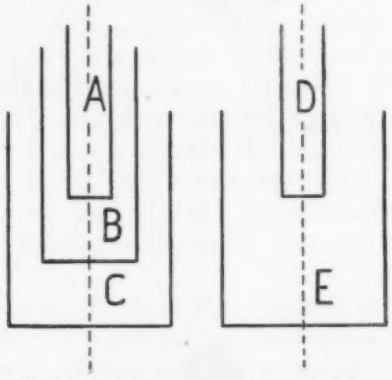

FIG. 1. COLORIMETER FOR TWO-COLORED INDICATORS

placed in *C*.. *A* is left empty.[6] Then, if the scale reads 70, the path of light along the left-hand dotted line passes through the alkaline form during 70 per cent of its path in the indicator, and through the acid form during 30 per cent. The light along the right-hand dotted line traverses an indicator solution in tube *E*, again of the same strength, and over a path equal in length to the

[6] For use in the determination of pH, a tube containing unknown solution without indicator can be slipped into tube A in order to compensate for color or turbidity without lengthening the apparatus unduly. In this case, water would be introduced into D to equal height in order to equalize absorption and the meniscus effects.

total path on the left. The merit of the instrument consists in the fact that the length of this total path is not affected by the motion of tube *B*, though the percentages of the path lengths in the two solutions are varied directly thereby. The indicator solution in tube *E* consists of a buffer mixture (or solution, the pH of which is to be determined) to which the proper amount of indicator has been added. If conditions are such that 70 per cent of the molecules encountered along the path on the right are in the alkaline modification, and 30, in the acid, then the eye will perceive identical impressions upon looking through the two systems from above. This will be the case, even if each modification is not pure, but admixed with the other,[7] or if each modification absorbs to some extent like the other, or if the indicator exhibits dichromatism. Consequently the apparatus may be used to determine the apparent percentage transformation of the indicator at different hydrogen-ion exponents; the relation being studied at different temperatures and subsequently being used to determine unknown hydrogen-ion exponents.[8]

To control the temperature of the buffer solutions or of the unknown solution, water can be circulated in a jacket (not shown in the figure) about the tube *E*. The temperature should be controlled to about one degree, or possibly better.

It is evident that titrations can be carried out in the tube *E*, a proper quantity of strong indicator solution being added for every cubic centimeter, or smaller unit, of added reagent.

[7] It need not be the case if the indicator is grossly contaminated with another indicator of different apparent dissociation constant, or if the indicator behaves like a dibasic or polybasic acid. Wegscheider (1915) has made statements equivalent to those in the text above.

[8] The instrument can of course be used at once and dependence put for the time being on the apparent dissociation constants and tables published (Gillespie, 1920). If the indicator used, the temperature, and what information as may be available as to the salt content of the solution, be recorded, the corrections can be applied at any time when better values for the indicators and other data are obtained. Although the writer can not admit that the method previously published or the use of a double colorimeter is to be classed as approximate because of doubtful optical assumptions, it is of course only approximate until precise calibration of the standards is made. At present the instrument is capable of giving more precision than could be obtained in the calibration made without it, and it may possibly disclose some small deviations from the simple dissociation curve.

It is well known that the simple law used in ordinary colorimetry, namely—the thickness of the solution times the concentration equals a constant when the thickness and concentration are varied in such a way as to match a standard color—does not hold for solutions of potassium dichromate. Indeed, with a color standard of different composition from the solution itself, the colors shown by a solution of potassium dichromate, as it is progressively diluted, can not be matched either by dilution of the color standard or by changing the depth of the layers. On the other hand, the changing colors can be matched in the double colorimeter. For standards (in tubes *B* and *C*), may be used a highly acid solution of potassium dichromate, and a solution of potassium (yellow) chromate. As the solution in question is diluted, it becomes necessary to change the ratio of the path lengths through the red and the yellow "forms", as well as to increase the path length through the solution (in tube *E*). It is generally assumed that a change of ionization occurs when potassium dichromate solution is diluted; and there seems to be no reason to doubt that the usual law of absorption holds for the *constituents* of the solution. There would appear to be no ground for a suspicion that the "dichromatism" of the sulphonephthalein indicators may interfere with their use in the double colorimeter.

In fact, to derive the law upon which ordinary colorimetry is based, we assume that light passing through a solution is affected independently by each particle of colored material, these particles usually being alike in kind. In order to apply the law to double colorimetry, we need only the further assumption that the same is true when the particles are not alike in kind, and it appears difficult to doubt this in the given case. Consideration of the expression for the intensity of the emergent light: Ia^{ec}, where I is the intensity of the entering light, a is the fraction absorbed by each particle, e is the thickness, and c is the concentration of particles, leads to the following conclusions.[9]

[9] The expression is applied to the different wave-lengths entering, the constant a being assumed different for each wave-length.

The variation of the constant a with wave-length, which leads to the dichromatism of the two-colored indicators, does not lead to any difficulty in the case of the double colorimeter. Dichromatism leads to the detection of errors made when turbidity of the solution to be measured is balanced optically in the usual manner. With a one-colored indicator the error made is no less because of the absence of dichromatism, but the error is not disclosed. In routine work, white light is advisable as a source for comparisons, when it can be used, so that such error may be made evident by dichromatism. When the subjective difficulties become too great for the use of white light, a screened light (Clark and Lubs, 1917) may be a valuable means of obtaining an approximate result.

SUMMARY

The recently published tables of Medalia are in disagreement with other published data and are not correct.

A colorimeter for two-colored indicators is described for use in accurate study of the indicators and for the measurement of hydrogen-ion exponent. The optical assumptions underlying its use are practically the same as those upon which ordinary colorimetry is based.

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THE EFFECT OF PEPTON UPON THE PRODUCTION OF TETANUS TOXIN

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Not very long after the outbreak of the European War, laboratory workers realized that a substitute for Witte pepton in bacteriological work would have to be obtained. Many laboratories were sufficiently stocked with this pepton for the first twelve to eighteen months, but in the spring of 1915 it was evident that the supply of Witte pepton in the United States was fast dwindling and that it would soon be unobtainable.

WITTE PEPTON

At the Bureau of Laboratories we had been using a Witte pepton glucose veal broth[1] with extremely satisfactory results. Those who have had experience in toxin production, particularly tetanus toxin, know that from time to time there will be marked variation in toxicity due to some unknown factor or factors. The same care may be used in the preparation of different lots of broth as well as in the filtration of the toxin, but the results may differ widely. In consideration of this fact it may be worth while to give the different potencies of the toxin made during the last three to four years when using Witte pepton glucose veal broth.

It is the opinion of some workers that the time of year has a definite influence upon the production of potent toxin. The results as given above show that the seasonal factor was of negative importance as far as toxicity was concerned. If we compare the quarterly results for the year 1915 we find that the averages did not vary greatly. The lowest average toxicity of 1:20,000

[1] Wilcox, Harriet Leslie, 1916, Jour. of Bact., 1, 333.

TABLE 1

Witte peptone

	1914	1915	1916	1917	1918
First quarter	None produced	7 lots 1:25,000 2 lots 1:15,000† 1 lot 1:10,000* 1 lot 1: 5,000 Average toxicity 1:20,000 Excluding*† average toxicity 1:22,000	1 lot 1:35,000 2 lots 1:30,000 2 lots 1:25,000 1 lot 1:20,000 2 lots 1:15,000 1 lot 1:13,000 1 lot 1:10,000§ 1 lot Below 1:5,000** Average toxicity 1:20,000 Excluding§** average toxicity 1:22,000	None produced	1 lot 1:25,000 1 lot 1:45,000 Average toxicity 1:35,000
Second quarter	None produced	5 lots 1:30,000 4 lots 1:25,000 1 lot 1:20,000 Average toxicity 1:27,000	4 lots 1:35,000 1 lot 1:10,000 1 lot 1: 5,000* Average toxicity 1:19,000 Excluding* average toxicity 1:22,000	None produced	None produced

Third quarter	2 lots 1:25,000	1 lot 1:40,000 2 lots 1:30,000 6 lots 1:25,000 3 lots 1:20,000 3 lots 1:15,000 2 lots 1:10,000 1 lot Below 1:5,000‡ Average toxicity 1:20,000	1 lot 1:30,000 3 lots 1:25,000 1 lot 1:20,000 2 lots 1:15,000	1 lot 1:100,000†† 1 lot 1: 60,000 1 lot 1: 25,000 2 lots 1: 20,000	None produced
	Average toxicity 1:25,000	Excluding‡ average toxicity 1:22,000	Average toxicity 1:20,000	Average toxicity 1:45,00	
Fourth quarter	1 lot 1:40,000 5 lots 1:25,000	2 lots 1:35,000 5 lots 1:25,000 1 lot 1:15,000 2 lots 1:10,000 2 lots 1: 5,000	1 lot 1:25,000 1 lot 1:25,000	1 lot 1:35,000 1 lot 1:20,000	None produced
	Average toxicity 1:27,000	Average toxicity 1:20,000	Average toxicity 1:25,000	Average toxicity 1:27,000	

* Flasks contaminated.

† Temperature of incubation down to 32°C.

‡ Broth very dark, due probably to over heating of glucose.

§ Broth very acid (2.2) after sterilization.

** Two lots of toxin were interchanged through error when removed from the incubator. Lot inoculated 1-26-16 was mixed with that of 2-2-16.

†† This preparation is referred to in text as lot 127.

occurred in the first, third and fourth quarters, while the second quarter gave an average toxicity of 1:27,000.

The differences in the quarterly averages of the year 1916 were so slight that they were scarcely worth mentioning. The third quarter in this period showed the lowest average toxicity of 1:20,000 while the first, second and fourth quarters gave an average toxicity of 1:22,000.

Doubtless if Witte pepton veal broth had been used throughout the year 1917 we should have had a greater variation in the quarterly averages, due to the two extremely potent toxins produced in the third quarter. As far as was known no especial care was taken in the preparation of these two broths nor in the filtration of these toxins. In lot 127, the usual procedure of testing for 1:15,000 and 1:25,000 was carried out. When the pigs succumbed in less than forty-eight hours, higher tests, namely 1:40,000 and 1:60,000 were made with similar results. Dilutions of 1:80,000 and 1:100,000 were then injected into guinea pigs weighing 350 grams each. The pig receiving the 1:80,000 dilution died between the second and third day, while the pig which received the 1:100,000 dilution succumbed on the fourth day. Since this was the strongest toxin ever obtained at the Bureau of Laboratories, tests were made of the toxin filtrate to preclude any possibility of spores or bacilli having passed through the Berkefeld filter. These tests were negative.

DIFFERENT PEPTONS

In the spring of 1916, we anticipated the present shortage of Witte pepton and made comparative tests with some of the domestic peptons on the market. These results are to be regarded as comparative only, but in those that were controlled by the use of Witte pepton, the differences in the toxicity, with one or two exceptions, were very significant.

A subsequent test, made with another preparation of Fairchild pepton, gave a toxicity of only 1:8000. No control test was made as we were unable to obtain Witte pepton.

TABLE 2

Comparative tests of different peptons

TEST	NAME OF PEPTON	TOXICITY
1	Witte (control)	1:25,000
	Leitz	1:5,000
	Eimer and Amend	1:1,000
2	Witte (control)	1:25,000
	Squibb	1:5,000
	Research no. 2	1:15,000
3	Witte (control)	1:25,000
	Fairchild	1:20,000
	Research no. 2	1:20,000
4	Witte (control)	1:30,000
	Parke, Davis and Company	1:8,000

MARTIN PEPTON

Sporadic attempts at using Martin pepton broth[2] were made early in the year 1916 with indifferent results. Nothing more was done until January, 1917, with regard to the adoption of Martin broth for tetanus toxin as used at the Pasteur Institute. The entire absence of Witte pepton at this time prevented us from making control tests with the usual Witte pepton glucose veal broth.

The highest average for this period, i.e., in the year 1917, occurred in the second quarter, when the average toxicity was 1:17,000. The first and third quarters gave respectively the average toxicity of 1:10,000 and 1:9000 while the fourth quarter gave the lowest average toxicity of 1:5,000.

Three different strains of *Clostridium tetani* were used for a comparative test (table 4). The strain designated "Research" is the culture used in the routine production of tetanus toxin at the Bureau of Laboratories. This strain was obtained about ten years ago from the New York State Laboratory at Albany but its origin and date of isolation are not known. Unless otherwise

[2] Martin, Louis. Annales de l'Institut Pasteur, vol. 12, p. 26, 1897.

TABLE 3

Martin pepton veal broth

	1917	1918
First quarter....	2 lots 1:15,000 1 lot Below 1:5,000 Average toxicity 1:10,000	2 lots below 1:5,000 Average toxicity below 1:5,000
Second quarter..	1 lot 1:25,000 1 lot 1:10,000 Average toxicity 1:17,000	1 lot below 1:5,000 Average toxicity below 1:5,000
Third quarter...	1 lot 1:15,000 2 lots 1:8,000 1 lot 1:5,000 Average toxicity 1:9,000	None produced
Fourth quarter.	1 lot 1:15,000 2 lots 1:8,000 3 lots 1:5,000 3 lots Below 1:5,000 Average toxicity 1:5,000	None produced

TABLE 4

Comparative tests of different strains in Martin pepton veal broth

	STRAINS	TOXICITY
Experiment 1. December 23, 1916.....	Research	1:35,000
	Goadby	1:35,000
	Pasteur	1:25,000
Experiment 2. May 19, 1917.....	Research	1:10,000
	Goadby	1:15,000
Experiment 3. December 15, 1917.....	Research	1:8,000
	Pasteur	1:8,000
	Pasteur	1:8,000

stated this was the strain used in our tests. The Goadby strain was obtained from Dr. MacConkey, Lister Institute, England, in 1916, and had been isolated from a war case by Dr. Goadby in

1915. The Pasteur strain was the one formerly in use at the Pasteur Institute, Paris, for the production of tetanus toxin. That this strain did not produce so potent a toxin (1:25,000) as the Research and Goadby strains (1:35,000) was somewhat surprising as it had been kept on a medium made with Martin pepton for years, whereas the Goadby and Research strains had been accustomed to this medium for several months only.

That Martin pepton broth as made by us is capable occasionally of giving potent toxin, is evidenced by the results obtained in experiment 1 (table 4) and in experiment 4 (table 5.) The other preparations of toxin broth were made with the same precautions and care but, as is shown, with far different results. When it was apparent that equal parts of Martin pepton and veal infusion were not giving satisfactory toxin, tests were made with broth prepared by using more concentrated Martin pepton solution with ordinary veal infusion (experiment 4, table 5); also by using the usual amount of Martin pepton solution with a more highly concentrated veal infusion. In neither case was the result so satisfactory as with the usual preparation. Subsequent broths were prepared according to the original method, that is to say using equal parts of infusion and pepton, but the toxin was far too low in potency to be used.

Word was received in January, 1918, with regard to the modification of Martin pepton broth used with favorable results for tetanus toxin at the Pasteur Institute. The variation between this procedure and the one we were using lay in the concentration of the pepton solution, in the shortened incubation period and also in the absence of glucose. A preparation of toxin broth (see experiment 6, table 6), was made according to this modification, one-half of the broth having 1 per cent glucose added to it while the other half was prepared without any sugar. After seven days incubation instead of the usual period of fifteen days, the cultures were filtered and tested. The results of the animal tests were most disappointing as pigs inoculated with 1 cc. of a dilution of 1:5,000 from each preparation showed an absence of tetanic symptoms. In his article on the preparation of this pepton, Martin advises that not less than five stomachs should be used

TABLE 5

Comparison of different methods for the preparation of Martin pepton veal broth

	MEDIA	STRAIN	TOXICITY
Experiment 4. June 13, 1917...	*Control broth* 1 part Martin pepton solution (200 grams of minced stomach to 1000 cc. H_2O) 1 part veal infusion (500 grams of veal to 1000 cc. H_2O)	Research	1:25,000
	Experimental broth 1 part of Martin pepton solution 1 part of veal infusion (500 grams of veal to 500 cc. H_2O)	Research	1:8,000
	Experimental broth 1 part of Martin pepton solution (400 grams of stomach to 1000 cc. H_2O) 1 part of veal infusion	Research	Below 1:5,000
Experiment 5. October 10, 1917	*Control broth* 1 part Martin pepton 1 part of veal infusion	Research	1:5,000
	Experimental broth 1 part of Martin pepton solution 2 parts of veal infusion	Research	1:8,000
	Experimental broth 1 part of Martin pepton solution (200 grams stomach to 1500 cc. H_2O) 1 part of veal infusion	Research	1:5,000
Experiment 6. April 2, 1918....	1 part of Martin pepton solution (300 grams of stomach instead of usual 200 grams to 1000 cc. H_2O) 1 part of veal infusion To the above mixture of pepton and veal infusion 1 per cent glucose was added	Research Research	Below 1:5,000
	1 part of Martin pepton (300 grams of stomach to 1000 cc. H_2O) 1 part of veal infusion. No glucose	Research	Below 1:5,000

TABLE 6

Berna pepton

	1918	1919	1920
First quarter...	None produced	1 lot 1:40,000 1 lot 1:35,000 1 lot 1:30,000 1 lot 1:25,000 1 lot 1:20,000 5 lots 1:15,000 4 lots 1:10,000 4 lots 1:8,000 1 lot 1:4,000 Average toxicity 1:15,000	1 lot 1:35,000 1 lot 1:30,000 1 lot 1:15,000 1 lot 1:10,000 Average toxicity 1:22,000
Second quarter.	1 lot 1:100,000 1 lot 1: 45,000 Average toxicity 1:72,000	1 lot 1:40,000 4 lots 1:35,000 1 lot 1:30,000 4 lots 1:25,000 2 lots 1:15,000 1 lot 1:10,000 3 lots 1: 5,000* 1 lot 1: 5,000 Average toxicity 1:21,000 Excluding* average toxicity 1:25,000	1 lot 1:25,000 1 lot 1:20,000 1 lot 1:5,000 Average toxicity 1:16,000
Third quarter..	2 lots 1:8,000 2 lots 1:5,000 Average toxicity 1:6,500	2 lots 1:60,000 Average toxicity 1:60,000	1 lot 1:20,000 2 lots 1:12,000 Average toxicity 1:14,000
Fourth quarter.	1 lot 1:60,000 1 lot 1:45,000 1 lot 1:35,000 1 lot 1:25,000 1 lot 1:12,000 1 lot 1: 7,000 Average toxicity 1:30,000	1 lot 1:30,000 1 lot 1:20,000 1 lot 1:15,000 Average toxicity 1:21,000	Production for this quarter not completed

* These three lots were grown at too high temperature, i.e., 42°C.

owing to the great variation of the different stomachs. We are accustomed to make about fifteen liters of pepton solution for one preparation of toxin broth. This amount requires seven or eight stomachs according to the size. At the Pasteur Institute, marmites or large casseroles holding not less than 60 liters are employed for the digestion of the pigs' stomachs.

BERNA PEPTON

About this time our attention was called by Dr. Noble of the New York State Laboratory to a pepton put on the market by the Swiss Vaccine and Serum Company, of Berne, Switzerland. The statement that this pepton was made according to Witte's recipe was received with some skepticism but the thought did occur to us that this might be a means of getting Witte pepton into the Allied countries.

A small amount of broth (about 15 liters), was made up according to our usual method using the "Berna" pepton instead of the Witte. So interested were we in the result, that a small amount was withdrawn from one of the flasks, filtered and tested on the sixth day of growth. The minimum lethal dose was found to be over 1:10,000 after even this short incubation period. At the end of fifteen days the rest of the cultures were filtered and tested. The pig which received 1 cc. of a dilution of 1:45,000 died of tetanus on the fourth day. A second preparation of toxin broth made with this pepton yielded a toxin of 1:100,000 in potency.

Broth made with Berna pepton has been used at the Bureau of Laboratories since May, 1918, to the present time with favorable results for the production of tetanus toxin (see table 6). The variations in the quarterly averages were greater than when Witte pepton was employed.

The average toxicities as given in this table showed again that the seasonal factor was of no importance in the production of potent toxins. The second quarter in the years 1918, 1919 and 1920 gave averages of 1:72,000, 1:21,000 and 1:16,000 respectively. The third quarter of these same years showed still

greater differences. The average toxicity in this period of 1918 was 1:6,500. In the year 1919 this quarter gave a high average toxicity of 1:60,000 and the same period in the year 1910 showed an average toxicity of 1:14,000.

SUMMARY

In going over the above results, it will be seen that Witte pepton has been an important factor in the production of a fairly constant potent tetanus toxin. The indications are that the Berna pepton is a satisfactory substitute for Witte since the latter is no longer available.

It would seem either that the Swiss Serum and Vaccine Company was justified in its claim that Berna pepton was made according to Witte formula or that Berna pepton was in reality Witte pepton since only when using these two peptons did we obtain a toxicity of 1:100,000.

It was a great disappointment that Martin pepton broth as made by us proved so unreliable. It is just possible that one of the reasons for the better results with this broth at the Pasteur Institute, is due to the fact that a larger number of stomachs are used for the preparation of the pepton than we can handle at one time at the Bureau of Laboratories.

ON THE GROWTH AND THE PROTEOLYTIC ENZYMES OF CERTAIN ANAEROBES

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In a work that soon will be published one of us, Blanc has extensively studied the biochemistry of certain anaerobes, especially *Bacillus sporogenes* and *Bacillus histolyticus*. It seemed to be of interest also to introduce in that work the methods, especially worked out in America by Clark and Lubs (1917) and their followers, for studying the influence of hydrogen ion concentration in its relation to growth and proteolytic activity. The other of us, Dernby, has already in a series of papers used these methods (Dernby 1918, Dernby and Avery 1918 and Dernby and David 1920), and as we have limited this paper to as short a communication as possible, we may for the description of methods refer to these papers mentioned above. What will follow are merely the main results of our work with some anaerobic microorganisms.

Some time ago, Wolf and Harris and Wolf and Telfer (1917) studied the biochemistry of the anaerobes, *B. welchii* (*perfringens*) and *B. sporogenes* (Metchnikoff), taking into consideration the influence of hydrogen ion concentration. It seems, however, that they, like many other authors at that time, paid too much attention to what is called "the limiting hydrogen ion concentration" and the influence of organic acids on the growth of the microörganisms. In our opinion the first thing to do, when studying the biochemistry of a given microörganism, is to determine the limiting and the optimal pH values for the growth of this organism in a given medium. We by no means claim that either of these values should be used as a new basis of classifying

the microörganisms, and especially as to the "limiting pH value" we are very doubtful whether it bears any deciding significance. The essential thing is to determine the optimum, and, even if this should change a little under different conditions, to fix such an initial reaction of the medium as can easily be reproduced and allow a sufficient growth.

In the first part of this paper we have determined the relation between growth and hydrogen ion concentration for a certain number of anaerobes, and in the second part we have studied the proteolytic activity of filtrates from *Clostridium sporogenes* and *Clostridium histolyticum*.

I. THE OPTIMAL HYDROGEN ION CONCENTRATION FOR THE GROWTH OF SOME ANAEROBES

The following microörganisms have been studied:
Clostridium sporogenes A (Klein-Metchnikoff)
Clostridium sporogenes O, isolated from horse excrements
Clostridium canadiense, isolated by Blanc from a case of gangrene
Clostridium histolyticum (Weinberg-Seguin)
Clostridium putrificum (Bienstock)
Clostridium perfringens (Veillon-Zuber)

For all these microörganisms the same broth medium was used. This was made up in the following way: 1 kgm. finely chopped veal was immersed in 2 liters of tapwater and allowed to autolyze at 37° for twenty-four hours; thereafter boiled and filtered; 0.5 per cent NaCl and 1 per cent pepton added; then sterilized at 110° for twenty minutes. In preparing an experiment the same amount of broth was divided among nine flasks to which different amounts of HCl or NaOH were added, in order to obtain certain hydrogen ion concentrations. Table 1 shows the composition of our nine standard media.

The contents of the nine flasks were then divided up into test tubes, 10 cc. of the same standard in each, and the test tubes then sterilized as before. Thus we could always work in a uniform manner. The tubes were allowed to stand at least for a day at room temperature in order to obtain a stable hydrogen ion con-

centration. All sets of nine tubes, except one intended to serve as a control for the pH determination, were inoculated with the same amount of bacterial suspensions. Simultaneously there were added a few milligrams of solid calcium sulphide powder. The microörganisms were taken from a twenty-hour pepton-gelatin culture. Before inoculation all tubes were heated to about 37°.

TABLE 1

Composition of "Standard" broth media

30 cc. broth, HCl, NaOH, or H_2O, 32 cc.

NUMBER	N. NaOH	N. HCl	H_2O	pH 24 HOURS AFTER STERILIZATION
	cc.	cc.	cc.	
1		1.8	0.2	3.1
2		1.0	1.0	4.0
3			2.0	4.9
4	0.3		1.7	6.0
5	0.6		1.4	6.5
6	0.9		1.1	7.0
7	1.2		0.8	7.5
8	1.4		0.6	8.0
9	1.8		0.2	8.8

The tubes were placed in an incubator at 37° and after certain intervals growth was recorded. No microscopic count of the bacilli was made, the growth was merely estimated, and in order to be able to reproduce the results graphically we have indicated the amount of growth by numbers from 0 to 4. Preliminary experiments indicated that a time of incubation of from fifteen to twenty hours was the most favorable to determine the optimal growth.

The initial and the final pH values were determined according to Clark and Lubs, with the method described in the mentioned paper by Dernby and Avery. All our experiments showed that under the chosen conditions (see tables 2 and 3) these anaerobes did not (at least during the first days of incubation) change the hydrogen ion concentration of the medium to any appreciable extent. Therefore it was not necessary here as in the case with pneumococcus or diphtheria bacillus to add any buffers. From

many aspects this is undesirable. Already earlier authors have pointed out that these anaerobes do not change the acidity or alkalinity of the medium.

TABLE 2

Growth of Clostridium sporogenes O.

Time of incubation, sixteen hours. Temperature 37°

NUMBER	pH INITIAL	pH AFTER 16 HOURS	DEGREE OF GROWTH
1	3.1	3.3	Trace
2	4.0	4.0	Trace
3	4.9	5.0	1
4	6.0	6.0	3
5	6.5	6.5	4
6	7.0	7.0	4
7	7.5	7.5	3
8	8.0	7.9	2
9	8.8	8.5	0

TABLE 3

Growth of Clostridium histolyticum

Time of incubation, sixteen hours. Temperature, 37°

NUMBER	pH INITIAL	pH AFTER 16 HOURS	DEGREE OF GROWTH
1	3.1	3.1	0
2	4.0	4.0	0
3	4.9	5.0	0
4	6.0	6.0	2
5	6.5	6.5	3
6	7.0	7.0	4
7	7.5	7.5	4
8	8.0	7.8	3
9	8.8	8.6	0

In tables 2, 3 and 4 some of our experiments are recorded. Our method, though apparently arbitrary, and giving no absolute basis for the calculation of the rate of growth, yet provides by the range of comparison which it affords, a quite adequate standard for all practical purposes.

In figure 1 the results from the tables 2, 3 and 4 are graphically represented. Even if the point for the optimum pH and the limits in the different cases change, we may as a whole state that

the curves of growth in relation to hydrogen ion concentration are almost identical for all the anaerobes studied. When comparing these curves with those given before for the pneumococcus and diphtheria bacillus (Dernby and Avery, 1918, Dernby and David, 1920), it is obvious that they are much broader, with limits from pH 5 to pH 9, whereas the limits for the pneumococcus are pH 7 to pH 8.3 and for the diphtheria bacillus pH 5.5 to pH 8. Obviously the hydrogen ion concentration has much

TABLE 4

Growth of four types of anaerobes

Strains used: 1. *Clostridium sporogenes A.* (Klein-Metchnikoff)
2. *Clostridium canadiense*
3. *Clostridium putrificum* (Bienstock)
4. *Clostridium perfringens* (Veillon and Zuber)

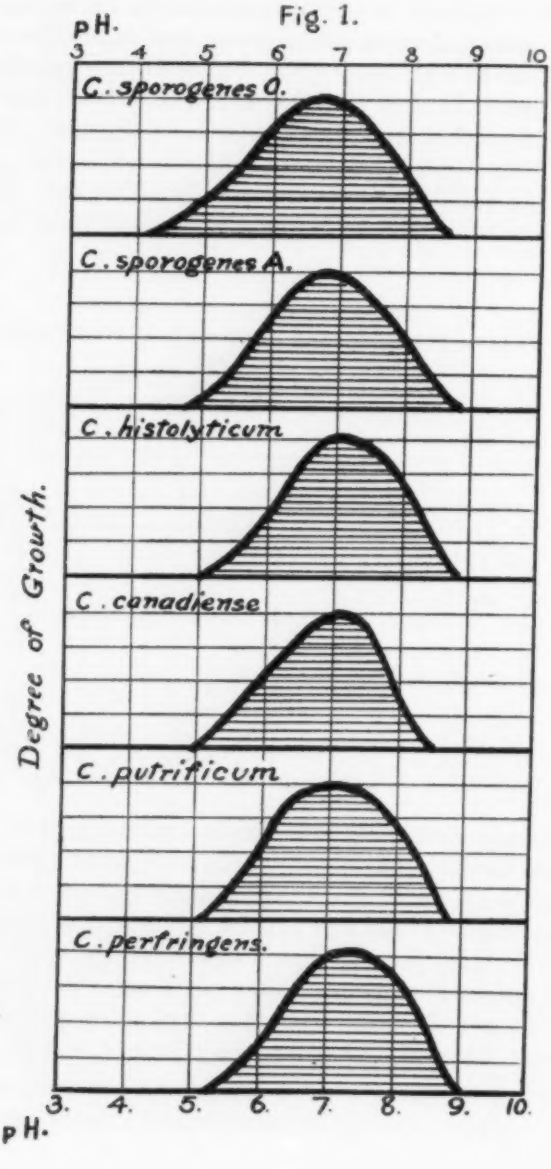
Time of incubation, seventeen hours. Temperature, 37°

NUMBER	pH INITIAL	DEGREE OF GROWTH			
		Clostridium sporogenes A	*Clostridium canadiensis*	*Clostridium putrificum*	*Clostridium perfringens*
1	3.1	0	0	0	0
2	4.0	0	0	0	0
3	4.9	Trace	0	Trace	Trace
4	6.0	2	2	1	1
5	6.5	4	3	4	3
6	7.0	4	4	4	4
7	7.5	3	3	4	4
8	8.0	2	1	3	3½
9	8.8	0	0	1	1

less influence—within certain limits—on the growth of these anaerobes than on the growth of, e.g., the pneumococcus.

The optimum for all of the microörganisms studied here seems to fall between pH 6.5 and pH 7.5. If any distinction should be made it might be said that *Clostridium sporogenes* seems to have an optimum a little less than pH 7 and the others a little more than pH 7. That indicates that these microörganisms grow most favorably in media which have a neutral reaction, that is pH 7.

Fig. 1.


pH.
3 4 5 6 7 8 9 10
C. sporogenes O.
C. sporogenes A.
C. histolyticum
C. canadiense
C. putrificum
C. perfringens.
Degree of Growth.
3. 4. 5. 6. 7. 8. 9. 10.
pH.

II. THE PROTEOLYTIC ACTIVITY OF FILTRATES FROM CLOSTRIDIUM SPOROGENES AND CLOSTRIDIUM HISTOLYTICUM

It is well known that anaerobes of this type are strongly proteolytic, dissolve fibrin and casein, liquefy gelatin, disintegrate pepton, and so on. The present mode of classifying the proteolytic enzymes seems to be to determine (1) the substrates attacked, (2) the products of digestion and (3) the optimal hydrogen ion concentration for the action. The two best substrates, which can be used in solution, are gelatin and pepton. In the cited paper by Dernby (1918) the method for using these substrates is fully described. Below we have studied the proteolytic activity of *Clostridium sporogenes* and *Clostridium histolyticum*. As enzyme the broth culture after passing a Chamberland filter has been used. By this method, we of course get information only in regard to the "ekto" enzymes, whereas the "endo" enzymes will escape our attention. The ideal thing would be to obtain large quantities of the bacilli, let them autolyze and determine the proteolytic activity of the autolysate. It is possible that in that case we should obtain enzymes both of the pepsin and of the trypsin-erepsin group as has been found is the case of yeast (Dernby, 1917).

The filtrates were obtained in the following way: For each culture 500 cc. of an ordinary broth made from autolyzed veal was taken, 1 per cent glucose and enough NaOH to render the initial reaction almost neutral (pH 7) were added. The mixture was sterilized at 107° for an hour. After cooling to 37° the flasks were inoculated with the microörganism in question, and simultaneously a minimal dose of solid calcium sulphide was added. The flasks were allowed to stand in the incubator at 37° for seventy-two hours, and then 3 cc. of chloroform were added. The culture was first filtered through paper and thereafter passed through a Chamberland filter. The clear filtrate was kept sterile at room temperature, and exhibited strong proteolytic activity for several months.

Gelatin tests

In order to make all experiments in exactly the same manner standard mixtures of gelatin and HCl or NaOH were made beforehand, of which the hydrogen ion concentrations were known. Table 5 shows the composition of these mixtures.

As our aim was to study the optimum reaction, it was sufficient for us to study the first stages in the liquefaction process. During this early period the hydrogen ion concentration will not change much, and buffers could be omitted.

TABLE 5

Composition of gelatin mixtures

The gelatin solution contained 14 per cent gelatin and 0.4 per cent thymol. 10 cc. gelatin, HCl, NaOH or H_2O, 12 cc.

NUMBER	N. HCl	N. NaOH	H_2O	pH
	cc.	cc.	cc.	
1	1.0		1.0	3.0
2	0.2		1.8	4.0
3			2.0	4.8
4		0.04	1.96	5.5
5		0.1	1.9	6.3
6		0.2	1.8	7.0
7		0.3	1.7	8.3

In preliminary experiments it was determined how much of the enzyme had to be taken in order to liquefy the gelatin within 4 to 20 hours at the optimum pH value 6.5.

In all cases a set of seven tubes was used, covering a range in pH from 3.0 to 9.0. Ordinarily 5 cc. gelatin were used for each test. The tubes were warmed to 37° before the enzyme was added. The gelatin contained 0.5 per cent thymol and the enzyme solution was aseptic. Therefore hardly any proteolytic activity from other microörganisms need be taken into consideration during the short time of digestion. A blank experiment was always run at the same time. After certain intervals the tubes were taken from the incubator and put into an ice bath for exactly ten minutes; thereafter the degree of liquefaction was estimated by the method given in the paper by Dernby (1918).

In figure 2 the results are graphically represented. The results from the tables 6 and 7 are almost similar. In both cases there is a marked proteolytic activity between pH 4 and pH 8, and the optimum is in both cases near pH 6. When allowed

TABLE 6

Clostridium sporogenes on gelatin

Filtrate from *Clostridium sporogenes* A. Klein-Metchnikoff. Temperature, 37°. 6 cc. gelatin, 0.5 cc. filtrate

NUMBER	pH INITIAL	DEGREE OF LIQUEFACTION AFTER		
		3 hours	6 hours	20 hours
1	3.0	0	0	0
2	4.0	0	0	1
3	4.8	0	½	2
4	5.5	1	1	5
5	6.3	2	3	5
6	7.0	1	2	3
7	8.3	0	0	1

TABLE 7

Clostridium histolyticum on gelatin

Filtrate from *Clostridium histolyticum* Weinberg and Seguin. Temperature, 37°. 6 cc. gelatin, 0.5 cc. filtrate

NUMBER	pH INITIAL	DEGREE OF LIQUEFACTION AFTER		
		2 hours	4 hours	20 hours
1	3.0	0	0	0
2	4.0	0	0	0
3	4.8	1	1½	4
4	5.5	2	4	6
5	6.3	1½	3½	6
6	7.0	0	1	5
7	8.3	0	1	5

to digest for a long period of time all tubes except the most acid ones are liquefied.

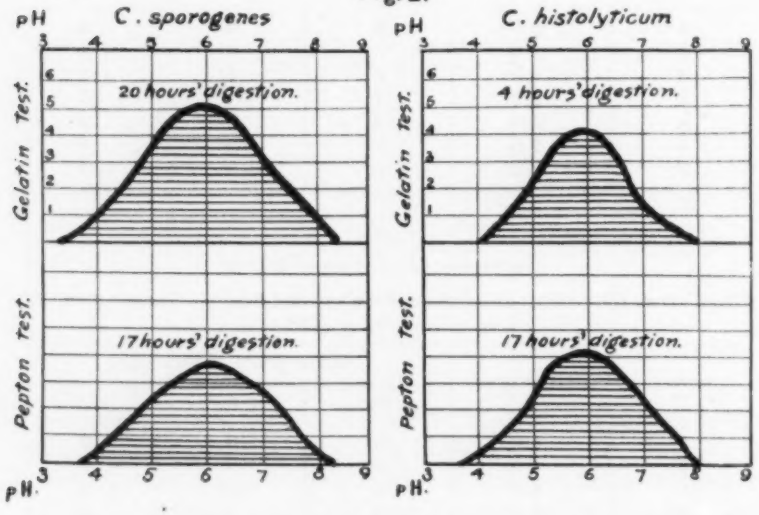
We seem justified in stating that in the filtrates from these two microörganisms enzymes resembling trypsin are present.

Pepton test

Table 8 shows the composition of the pepton standard solutions. The rate of digestion was measured with the Sörensen formol method. Tables 9 and 10 give the results of experiments with *Clostridium sporogenes* and *Clostridium histolyticum*.

It may be objected that in this case the Van Slyke method might have given sharper values, but the only question we wanted to study was the optimum for the action of the enzymes on pepton, and for this purpose the formol method gives results which

Fig. 2.



can not be misinterpreted. In figure 2 the results are graphically represented. It is evident that the enzymes from *Clostridium sporogenes* act in the same manner as to the pH optimum as those from *Clostridium histolyticum*. For both the optimum is at pH 6 and the range within which they act is pH 4 to pH 8. Also in this case the enzymes acting on pepton seem to be "tryptases."

From figure 2 it is evident that the action on gelatin and the action on pepton of the two anaerobes in relation to the hydrogen

TABLE 8

Composition of pepton mixtures

10 cc. 4 per cent pepton solution, NaOH, HCl or H_2O, 40 cc. Chloroform and toluene added

NUMBER	N. NaOH	N. HCl	pH
	cc.	cc.	
1		0.6	3.0
2		0.1	4.0
3			4.7
4	0.04		5.5
5	0.08		6.2
6	0.2		7.1
7	0.3		7.7
8	0.5		8.5

TABLE 9

Clostridium sporogenes A. on pepton

10 cc. pepton, 0.5 cc. filtrate. Temperature, 37°. Time of digestion, seventeen hours

NUMBER	pH INITIAL	LIBERATED AMINO-N IN 10 CC. AFTER 17 HOURS
		mgm.
1	3.0	0
2	4.0	0.1
3	4.7	0.55
4	5.5	0.65
5	6.2	1.0
6	7.1	0.65
7	7.7	0.4
8	8.5	0

TABLE 10

Clostridium histolyticum on pepton

10 cc. pepton, 0.5 cc. filtrate. Temperature, 37°. Time of digestion, seventeen hours

NUMBER	pH INITIAL	LIBERATED AMINO-N IN 10 CC. AFTER 17 HOURS
		mgm.
1	3.0	0
2	4.0	0.2
3	4.7	0.7
4	5.5	1.95
5	6.2	1.95
6	7.1	1.1
7	7.7	0.3
8	8.5	0

ion concentration are almost identical. Whether it is the same tryptase that acts in both cases, or whether there are several is impossible to say.

The conclusion we have arrived at by these experiments is simply that the proteolytic enzymes in filtrates from *Clostridium sporogenes* and *Clostridium histolyticum* seem to be very much alike, and that the enzymes which can be detected belong to the tryptase group.

It must be remembered however that in the living or dead microörganisms there are also present proteolytic enzymes of other types.

SUMMARY

The optimal and limiting hydrogen ion concentrations for the growth of the anaerobes, *Clostridium sporogenes*, *Clostridium histolyticum*, *Clostridium canadiense*, *Clostridium putrificum* and *Clostridium perfringens* have been determined. The range in which all of these organisms live has the limits pH 5 to pH 9. The optimum range for all seems to be at or about the neutral point pH 7 and is apparently a rather broad one.

2. The proteolytic activity of filtrates from *Clostridium sporogenes* and *Clostridium histolyticum* has been studied. Gelatin is liquefied and pepton is disintegrated in the range pH 4 to pH 8, and the optimum for both these reactions seems to be about pH 6. The conclusion is that in the filtrates a *tryptase* is present.

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